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AN ASSESSMENT OF PERIPHERAL NERVE DAMAGE IN THE RAT FOLLOWING NON-FREEZING COLD EXPOSURE: An Electrophysiological and Histopathological Examination

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TECHNICAL REVIEW AND APPROVAL
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The experiments reported herein were conducted according to the principles set forth in the current edition of the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This technical report has been reviewed by the NMRI scientific and public affairs staff and is approved for publication. It is releasable to the National Technical Information Service where it will be available to the general public, including foreign nations.

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The effect of exposure to non-freezing cold temperature on peripheral nerve was studied in vivo. Rats' tails, and a portion of their lower backs, were submerged in 1°C water for either 10 or 12 hours. Changes in evoked ascending nerve action potentials, and muscle action potentials in the rat tail and lumbar spine, were studied periodically over a three week period following cold exposure. In addition, ventral caudal nerves were excised 27 days following cold exposure and histopathology was performed. Electrophysiological analysis indicated initial nerve damage appeared to be just below the surface of the water, and later, in the first week after exposure, Wallerian degeneration occurred. Histopathological analysis revealed damage to the large myelinated fibers and capillaries within the fascicle following cold exposure. These results further validate the use of

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19. the rat tail as a model for non-freezing cold injury (NFCI) and suggest that the injury's etiology is multifaceted, which may require a variety of strategies and interventions to prevent its occurrence.

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Experiments reported herein were conducted according to the principles set forth in the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council, DHHS Publications (NIH) 86-23 (1985).

INTRODUCTION

The debilitating effect of non-freezing cold temperature on peripheral nerves of the extremities, particularly the hands and feet, has been a pervasive problem in military conflicts from the Crimean War, World Wars I and II, the Korean war, and, most recently, the Falklands war (1). Non-freezing cold injury (NFCI) often occurs after prolonged exposure of the limbs, especially the feet, to water temperatures ranging from just above freezing to 15°C. The injury was historically termed trench foot, or immersion foot, and demonstrates a sequela of symptoms, which includes not only peripheral nerve damage, but limb edema, skin blistering, hyperhidrosis, vascular damage, and tissue loss (1). This injury continues to be a threat to Naval and Marine personnel operating under cold, wet weather conditions.

To better understand and characterize NFCI, animal models have been used to study this phenomenon. Experimental studies using the rat-tail model have shown that, following extended exposure to cold water (1°C), the normal pattern of vasodilation to cold challenges is absent and thermal sensitivity is increased in the tail (2, 3). Since peripheral nerve damage is one of the major components of NFCI, this report will present electro-physiological results using the rat-tail model. In a recent report, Van Orden et al. (4) proposed a procedure for studying changes in ascending nerve action potentials (NAPs) in the rat tail. Recording electrodes were placed in the middle portion of the rat tail, lumbar spine, and somatosensory cortex, and NAPS were recorded following distal tail stimulation. Results from this study showed that, following cold exposure, ascending NAP amplitudes were reduced in the tail and somatosensory cortex, giving a clear indication that neural dysfunction had occurred. The present experiment extends and modifies this original design by using three tail sites (distal, middle, and proximal) and a recording site in the lumbar spine. This procedure allows for a more detailed account of the time course and pattern of neural dysfunction following cold exposure. In addition to recording ascending NAPs, muscle action potentials (MAPs) in the tail were also examined. Recording changes in MAPs are important because a common clinical symptom associated with NFCI is muscle weakness, and examining MAPs with this model will allow for a better understanding

of the neural dysfunction associated with this symptom. This report also presents histological analysis of ventral caudal rat-tail nerves following non-freezing cold exposure.

METHODS

Subjects: Four male Long-Evans rats maintained at 300-330 grams served. Animals were fed as needed, provided water ad libitum, and maintained on a 12-hr L/D cycle (lights on at 0600).

Apparatus: A TECA Neurostar electromyograph system was used to record and stimulate evoked responses. Rectangular stimulating pulses of 0.5 ms were used. Stainless steel needle electrodes were used for stimulation and recording.

Procedure: Figure 1 illustrates recording and stimulating sites for NAPs and MAPs. Ascending NAPs were recorded at two locations on the tail, t1 and t2, following stimulation from electrode pairs at t2 and t3. Evoked NAPs were also recorded from the cauda equina with subcutaneous electrodes at the dorsolumbar spine junction following stimulation from three sites in the tail -- t1, t2, and t3. In addition, MAPs were recorded at t3 following stimulation from t1 and t2. Sites t1, t2, and t3 were spaced 40 mm apart. At each site in the tail, recording and reference electrodes were spaced 10 mm apart. The lumbar spine recording site was 90 mm from t1, and the reference electrode was placed 20 mm rostral to the recording electrode. Nerve conduction was recorded prior to cold exposure; 1, 4, 7, or 8; 14 or 15; and 21 or 22 days following exposure to 1°C water. Prior to each recording session rats were anesthetized with pentobarbital (50 mg/kg), injected intraperitoneally.

Cold Exposure. For tail and lower back water immersion, rats were restrained in a plexiglass cylinder, 6.7 cm in diameter and 20 cm long. The floor of the cylinder was made of fiberglass mesh, with a 2-cm hole in the center to allow the rat's tail to protrude through. The bottom portion of the cylinder was lowered vertically into a circulating water bath maintained at 1°C until the rat's lower back was submerged (see figure 1 for approximate water level). Rats were exposed, continuously, for 10 (n=2) or 12 hrs (n=2).

Histology. Twenty-seven days following cold exposure rats were sacrificed, and the excised ventral caudal nerves of the rat tail were placed in a solution of 4% glutaraldehyde and .01% cacodylic acid and fixed overnight. The specimens were processed, cut into sections, and stained.

RESULTS

Electrophysiology

Figure 2 illustrates representative evoked potential tracings from each site prior to cold exposure and 1 and 4 and 8 days following a 10-hr cold exposure. Table 1 presents NAP and MAP velocities and amplitudes for each rat for the two cold exposure conditions. The pattern of NAP and MAP amplitude changes was similar in rats exposed for 10 or 12 hours. After cooling at 1°C, the major change within 24 hours was a diminution in the amplitude of the cauda equina potential with tail stimulation, the reduction being 65-100%. During this period NAP amplitude from the tail itself decreased, but this change was less marked (50-70%). Later in the next week there was further reduction in NAP amplitude in the tail itself (80-90%) and no further change in the cauda equina. Evoked MAP amplitudes in the tail showed a fall during the first week. With the possible exception of rat 82, nerve conduction velocity (NCV) showed little change following cold exposure.

Histopathology

Figures 3 and 4 show histological examples of ventral caudal rat tail nerves. Figure 3 shows a cross section of a normal nerve fascicle with a high density of myelinated fibers of various diameters within the endoneurium. The pale center is the axon and the dark rim represents the myelin. Endoneural capillaries (a) are present with dormant endothelium and a few red blood cells in the lumen. Figure 4 shows a cross section of a nerve fascicle 27 days following a 12-hr non-freezing cold exposure. Within the fascicle there is increased interaxonal space and a large reduction in myelinated axons. There is also evidence of degenerating axons showing a homogenized and swollen appearance and a loss of axomyelinic distinction (b). Other axons show peculiar grayish homogenous axoplasm (d). The smaller diameter fibers are partially preserved and are mostly located in the periphery (e). This pattern of

survival may indicate a vascular component in this most severe case of NFCI, since the capillaries within the endoneurium show prominent endothelial cells and quite a few have obliterated lumina (c).

DISCUSSION

These data suggest that when the rat tail is cooled for 10 to 12 hours at 1°C, the initial nerve damage is just below the surface of the coolant. Later, Wallerian degeneration occurred at the distal parts of the affected fibers in the tail. This pattern of nerve damage is similar to that reported in the rabbit limb after prolonged cooling (5, 6) and may be related to a local problem with axonal transport at the interface between cold and warm nerves. Preliminary histological analysis also indicated that the larger myelinated fibers were particularly affected by cold exposure, while the smaller fibers remained intact at the periphery of the fascicle, and the capillaries were severely damaged within the endoneurium. This result suggests nerve damage may be related to changes in supporting vasculature within the fascicle and possible nerve ischemia as well.

Previous research using animal models has shown changes in muscle and blood vessels following cold immersion (7, 8), suggesting that NFCI may be related to changes in supporting vasculature leading to nerve ischemia, while others have shown peripheral nerve degeneration without apparent damage to blood vessels (6, 9, 10). It appears that the mechanism of nerve damage by NFCI appears multifaceted and may depend upon the general procedure used to induce the cold injury. For example, in those experiments in which whole limbs were cooled and no change in blood vessels were found, animals were anesthetized during cold exposure. In this experiment and those of Blackwood and Russell, in which blood vessel damage is reported, the animals were conscious during cold exposure. It is possible that the added stress associated with the cold exposure condition in the awake, conscious animal could provide a component to NFCI that leads to blood vessel changes and nerve ischemia.

In summary, electrophysiological and histological data from the present experiment suggest that damage to peripheral nerves exposed to non-freezing cold may be related to a variety of changes both in the nerves themselves and in their supporting

vasculature. Therefore, treatments designed to prevent NFCI will have to consider both of these two potential causes of the injury.

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FIGURE LEGENDS

Figure 1. Diagram of stimulating and recording sites of neural evoked potentials in the rat tail and lumbar spine.

Figure 2. Representative evoked potential recordings for rat 85 prior to 10 hours of cold exposure and 1, 4, and 8 days following exposure. The number in the first panel of each row represents voltage per division on the Y axis. Time in msec is represented on the x axis. The first 6 rows are represented by the 2-msec legend, and the last two rows are represented by the 4-msec legend.

Figure 3. Cross section of a normal nerve fascicle showing myelinated fibers of various diameters (multiple stain solution, x 300). (a) = a normal endoneurial capillary.

Figure 4. Cross section of a 12-hr cold induced damaged nerve fascicle (multiple stain solution, x 300). B= degenerating axon, (c)= capillaries with obliterated lumina. (4)= axon with grayish homogenous axoplasm, (e)= smaller diameter axon.

Table 1.
12-hr Cold Exposure
Rat 81

Stimulation & Recording Sites	Pre-Exp.	Post Day 1	Post Day 4	Post Day 7	Post Day 14	Post Day 21
Ascending Lumbar Spine Action Potentials						
T1 to LS						
Amp. (μV)	12.0	3.7	2.4	4.7	4.8	5.0
NCV (M/sec)	69.2	62.1	64.3	64.3	56.3	62.1
T2 to LS						
Amplitude	5.5	0.8	0.7	1.5	1.6	0.8
NCV	51.8	49.1	51.8	50.0	50.0	50.0
T3 to LS						
Amplitude	1.9	0.00	0.00	0.00	0.00	0.00
NCV	39.0					
Ascending Tail Nerve Action Potentials						
T3 to T1						
Amp. (μV)	42.0	21.0	18.0	3.0	3.3	3.3
NCV (M/sec)	42.9	52.9	52.9	47.4	45.0	45.0
T3 to T2						
Amplitude	110.0	80.0	80.0	6.0	8.5	8.0
NCV	50.0	57.0	57.0	40.0	50.0	36.4
T2 to T1						
Amplitude	160.0	90.0	65.0	22.0	21.0	21.0
NCV	57.1	44.4	57.1	44.4	50.0	50.0
Motor Action Potentials						
T1 to T3						
Amp. (mV)	7.0	1.7	0.4	1.0	2.9	3.6
T2 to T3						
Amplitude	11.5	2.4	0.7	1.4	3.8	5.0
NCV						
(M/sec)						
T1 to T2	35.7	33.3	27.8	35.7	41.7	41.7

Table 1 con't.
12-hr Cold Exposure
Rat 82

Stimulation & Recording Sites	Pre-Exp.	Post Day 1	Post Day 4	Post Day 8	Post Day 15	Post Day 22
Ascending Lumbar Spine Action Potentials						
T1 to LS						
Amp. (μV)	15.0	0.9	3.6	3.1	1.8	3.4
NCV (M/sec)	69.2	52.9	52.9	52.9	56.3	56.3
T2 to LS						
Amplitude	7.0	0.00	1.3	1.3	0.6	1.1
NCV	63.6		46.7	46.7	51.9	50.9
T3 to LS						
Amplitude	3.5	0.00	0.00	0.00	0.00	0.00
NCV	61.3					
Ascending Tail Nerve Action Potentials						
T3 to T1						
Amp. (μV)	44.0	12.0	4.5	5.0	5.5	6.0
NCV (M/sec)	64.3	42.9	52.9	50.0	50.0	47.4
T3 to T2						
Amplitude	110.0	48.0	22.0	14.0	18.0	21.0
NCV	66.7	50.0	57.1	50.0	50.0	50.0
T2 to T1						
Amplitude	150.0	60.0	12.0	18.0	23.0	20.0
NCV	50.0	47.1	50.0	50.0	50.0	50.0
Muscle Action Potentials						
T1 to T3						
Amp. (mV)	7.0	0.0	0.0	0.04	0.06	0.23
T2 to T3						
Amplitude	9.0	0.17	0.0	0.06	0.10	0.23
NCV						
(M/sec)						
T1 to T2	62.5			31.25	31.25	31.25

Table 1 con't.
10-hr Cold Exposure
Rat 84

Stimulation & Recording sites	Pre-Exp.	Post Day 1	Post Day 4	Post Day 7	Post Day 14	Post Day 22
Ascending Lumbar Spine Action Potentials						
T1 to LS						
Amp. (μV)	15.0	3.0	2.5	2.3	3.0	3.0
NCV (M/sec)	75.0	56.3	64.3	60.0	56.3	60.0
T2 to LS						
Amplitude	6.0	1.0	1.1	0.8	1.0	1.2
NCV	63.6	58.3	56.0	53.8	53.8	53.8
T3 to LS						
Amplitude	2.1	0.0	0.0	0.0	0.0	0.0
NCV	54.3					
Ascending Tail Nerve Action Potentials						
T3 to T1						
Amp. (μV)	55.0	32.5	32.0	8.0	7.4	7.5
NCV (M/sec)	47.4	50.0	64.3	60.0	56.3	56.3
T3 to T2						
Amplitude	130.0	120.0	110.0	25.0	30.0	25.0
NCV	50.0	57.1	57.1	50.0	57.1	57.1
T2 to T1						
Amplitude	170.0	120.0	110.0	25.0	30.0	25.0
NCV	50.0	57.1	57.1	50.0	57.1	57.1
Muscle Action Potentials						
T1 to T3						
Amp. (mV)	6.5	3.8	1.3	0.9	1.5	1.7
T2 to T3						
Amplitude	9.0	4.6	1.7	1.2	1.6	2.1
NCV						
(M/sec)						
T1 to T2	35.7	41.7	35.7	41.7	41.7	41.7

Table 1 con't.
10-hr Cold Exposure
Rat 85

Stimulation & Recording Sites	Pre-Exp.	Post Day 1	Post Day 4	Post Day 7	Post Day 14	Post Day 22
Ascending Lumbar Spine Action Potentials						
T1 to LS						
Amp. (μV)	22.0	6.5	6.0	6.0	5.0	5.0
NCV (M/sec)	64.3	64.3	60.0	56.3	64.3	64.3
T2 to LS						
Amplitude	7.5	2.5	2.1	2.5	1.9	1.9
NCV	56.6	58.3	58.3	53.8	58.3	60.1
T3 to LS						
Amplitude	2.7	0.7	0.8	0.7	0.5	0.9
NCV	52.8	54.3	60.0	50.0	46.3	55.9
Ascending Tail Nerve Action Potentials						
T3 to T1						
Amp. (μV)	40.0	30.0	30.0	16.0	18.0	18.0
NCV (M/sec)	47.4	50.0	60.0	50.0	47.4	47.4
T3 to T2						
Amplitude	85.0	70.0	80.0	32.5	37.5	41.3
NCV	44.4	44.4	57.1	50.0	57.1	50.0
T2 to T1						
Amplitude	190.0	160.0	150.0	52.0	50.0	55.0
NCV	57.1	57.1	50.0	57.1	57.1	57.1
Muscle Action Potentials						
T1 to T3						
Amp. (mV)	2.7	0.6	0.6	0.5	0.6	0.8
T2 to T3						
Amplitude	3.0	0.7	0.7	0.6	0.8	0.9
NCV						
(M/sec)						
T1 to T2	41.7	50.0	41.7	35.7	31.3	31.3

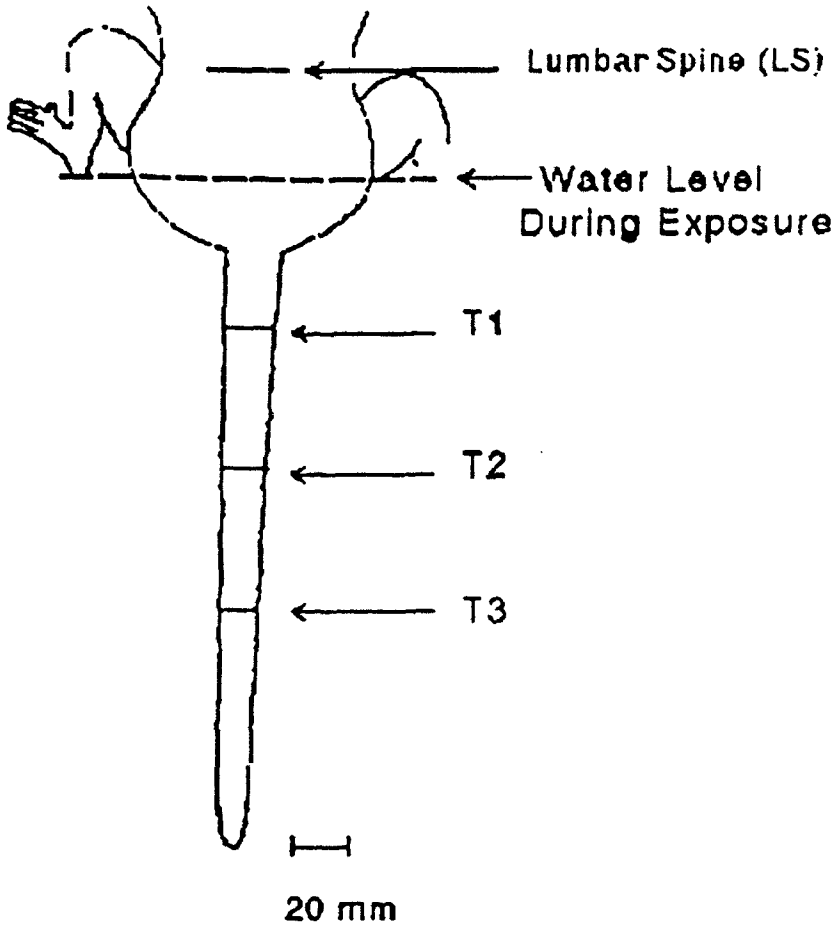
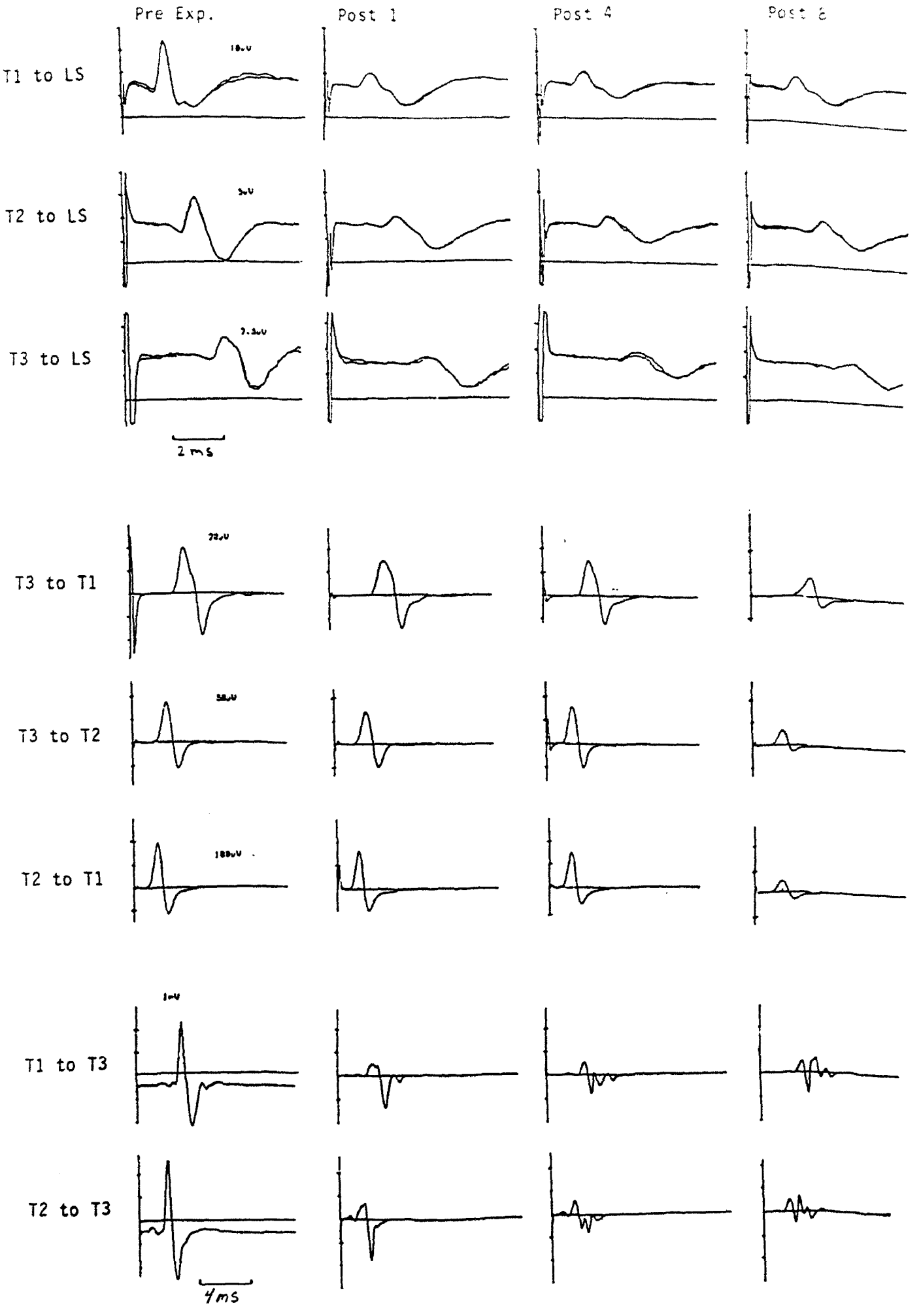


FIGURE 1


Pre Exp.
Post 1
Post 4
Post 6
T1 to LS
T2 to LS
T3 to LS
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T3 to T1
T3 to T2
T2 to T1
T1 to T3
T2 to T3
4ms

FIGURE 2

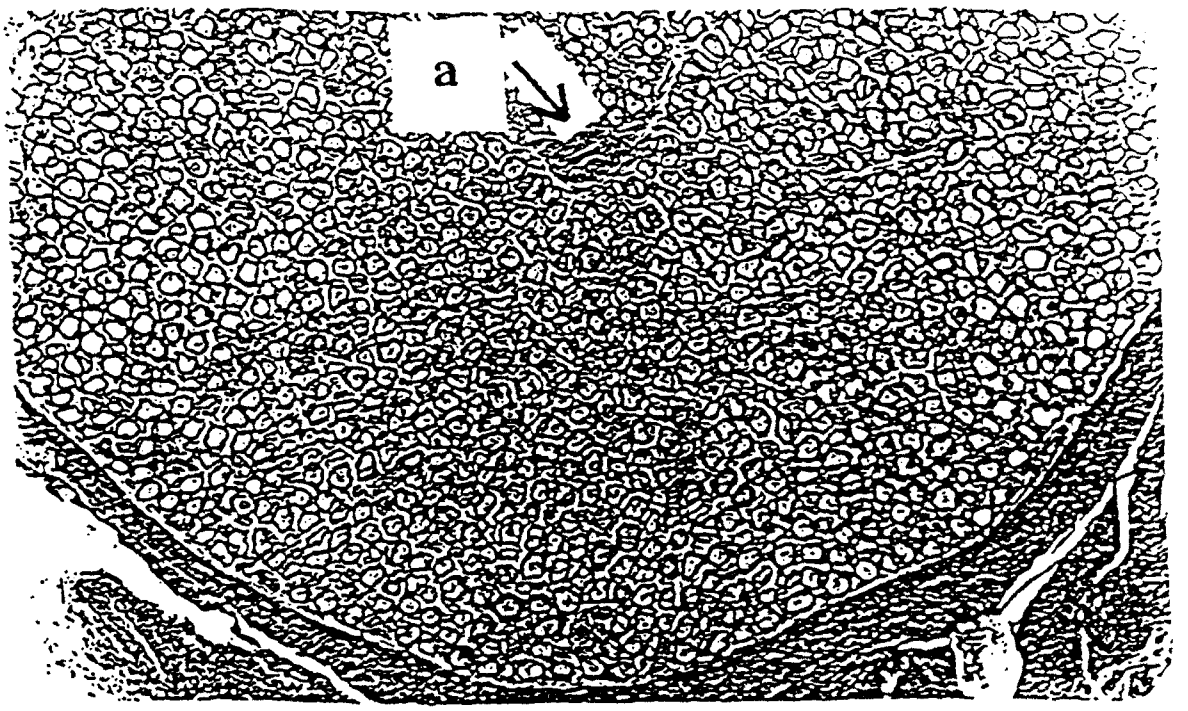

FIGURE 3

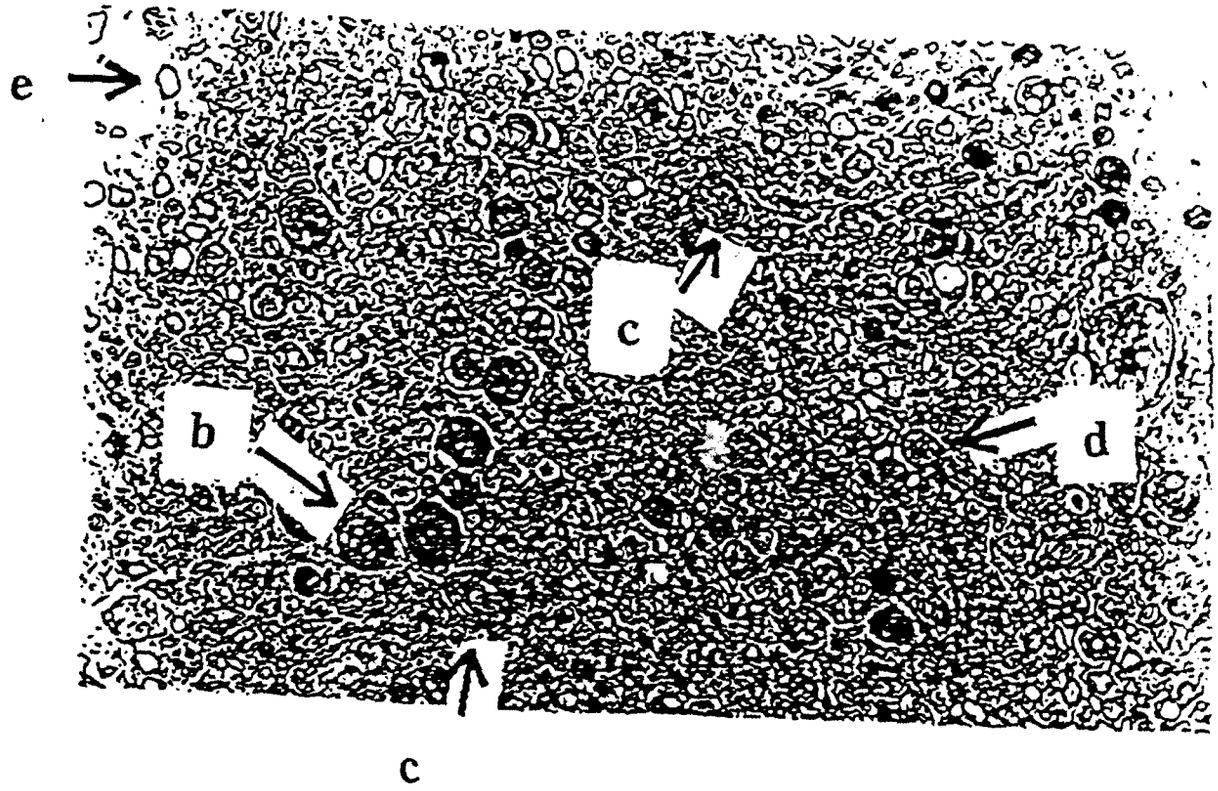

FIGURE 4